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MITOGENIC AND COLONY FORMING UNIT RESPONSES OF SPLEEN CELLS FRO--ETC(U)
SEP 76 G D LEDNEY, J V MONIOT, M R GAMBRILL

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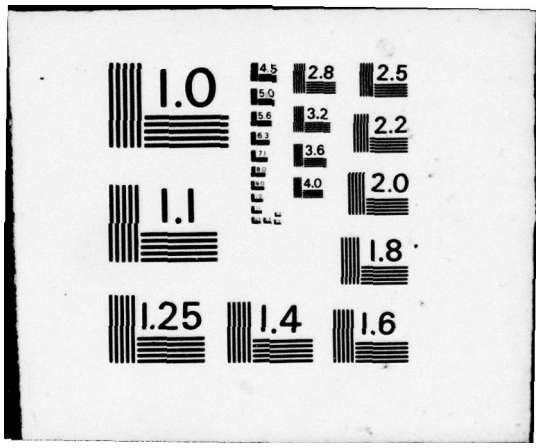
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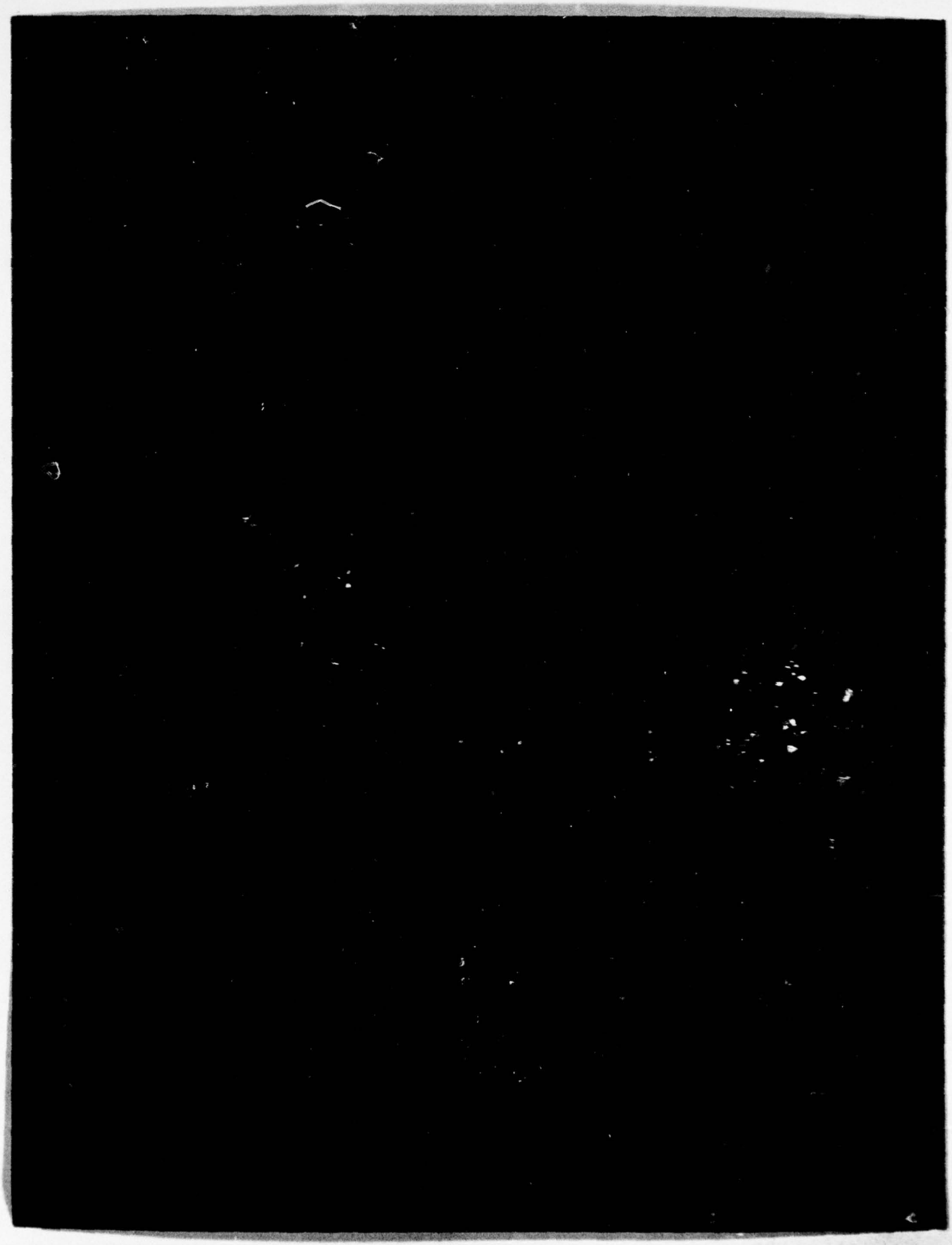

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The induction of malignant disease by radiation either alone or in combination with other severe injuries is a critical collateral damage problem. The mechanisms underlying the host's responses to malignancy are not well known. Thus, an integrated study of the host's hematocytopoietic responses to malignancy was undertaken with the aid of an animal model. Understanding and interpreting these responses is of critical value in determining (1) treatments that might obviate malignant cell induction

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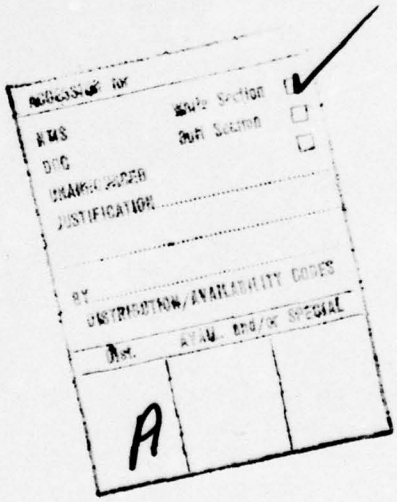
20. ABSTRACT (continued)

or (2) treatments that will assist in the cure of the malignancy. Certain non-leukemic, solid animal and human tumors not directly involved with the hematopoietic system are known to affect indirectly the various cellular compartments of the system. One such tumor, the Lewis lung (3LL) transplantable carcinoma, produces splenic enlargement in mice engrafted sc with a tumor cell inoculum. Thus, we hypothesized that the splenic enlargement was associated with myelocytopoietic and lymphocytopoietic changes that were dependent on (1) initial 3LL cell load, (2) time after engraftment with 3LL cells and (3) engrafted mouse strain. The hypothesis was tested by engrafting both C57BL/6 male and B6CBF1 male mice with either (1) 10^4, (2) 5×10^4 or (3) 2×10^5 viable 3LL tumor cells. Control treated mice received either (1) no tumor cells or (2) 2×10^5 irradiated 3LL cells. On days 3, 7 and 14 after sc engraftment the following responses were measured simultaneously in replicate experiments: (1) splenic enlargement and histologic appearance, (2) splenic lymphocyte responses to phytohemagglutinin and to (3) lipopolysaccharide and splenic cell colony forming unit potentials as measured by the (4) colony forming unit-spleen and (5) colony forming unit-culture assays. In all tumor cell engrafted mice, the splenic weight was greater than that seen in control untreated mice. In each strain, the splenic weight increase was dependent on the number of engrafted tumor cells and the time after engraftment. Hyperplasia of the reticuloendothelial system was observed at all tumor cell doses at all time intervals. Reticuloendothelial system hyperplasia tended to increase as a function of time and tumor cell dose. The incorporation of tritiated thymidine into splenic lymphocytes stimulated with phytohemagglutinin was reduced in all mice engrafted with tumor cells. There was a strain difference in response to this mitogen that was dependent upon the tumor cell load and time after tumor cell engraftment. Tritiated thymidine incorporation into splenic lymphocytes stimulated with lipopolysaccharide was increased in all mice engrafted with tumor cells. There was a difference in mouse strain responses to lipopolysaccharide, but no differences were associated with the various tumor cell doses and times after engraftment. The number of colony forming units-spleen was marginally increased and was dependent upon the dose and time of tumor cell administration and the mouse strain engrafted. Colony forming unit-culture growth of spleen cells obtained from tumor cell engrafted mice was increased at all time intervals and tumor cell doses tested. Generally, the colony forming unit-culture growth of the spleen cells of both mouse strains injected with tumor cells increased as the tumor cell dose and time increased. In conclusion, myelocytopoietic and lymphocytopoietic changes observed in the splenic cell populations of mice engrafted with the Lewis lung carcinoma were dependent on (1) initial tumor cell load, (2) time after engraftment with tumor cells and (3) engrafted mouse strain. The data support the contention that investigators using 3LL transplantable carcinoma systems designed to evaluate curability of primary tumors and/or lung metastasis by either chemotherapy, radiotherapy, or adoptive immunotherapy should consider the above observations when interpreting their data.

PREFACE

The authors wish to thank the scientific, technical and supportive personnel of the Armed Forces Radiobiology Research Institute for their assistance, advice and criticisms during the course of the work. The assistance of E. D. Exum, J. M. Sheil, W. E. Jackson III and A. N. Ledney is particularly acknowledged.



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INTRODUCTION

Tumor associated changes in hematocytopoietic organs have been found in humans and animals bearing certain solid tumors even though their malignancy does not directly involve such tissues.[7,10,12,16,18] The altered hematocytopoiesis can take the form of a leukemoid reaction which is characterized by granulocytosis and splenomegaly[8] and increased numbers of splenic and soft agar colonies derived from either the spleen or the bone marrow.[3,13,22,23] In addition, the reactivity of splenic cells to nonspecific mitogenic stimulation may be reduced in mice bearing various solid transplantable tumors.[11,15]

In mice bearing certain transplantable tumors, the spleen undergoes enlargement and thus becomes a focus of attention for investigations dealing largely with the immune responses of cells from that organ. To date, no studies have been reported whereby simultaneous examinations for lymphocytic and myelocytic proliferation were done. Such analyses would be expected to provide (1) insight into tumor associated hematopoietic disturbances and (2) better interpretation of hematopoietic dysfunctions observed in animals subjected to either radiation, chemical, or immunologic manipulative procedures designed to eradicate the malignancy.

In this laboratory, splenic enlargement was observed in mice bearing the Lewis lung (3LL) carcinoma. Thus, based upon this observation and that of others,[7,8,12,16,18] it was hypothesized that profound simultaneous alterations in lymphocytopoiesis and myelocytopoiesis would be observed in spleen cells derived from mice bearing the 3LL tumor. We observed simultaneous (1) decreases in lymphocyte responsiveness to phytohemagglutinin, (2) increases in lymphocyte responsiveness to lipopolysaccharide and (3) increased numbers of colonies formed on the spleen and in soft agar. Initial dose of tumor cells, time of spleen cell analysis after injection with tumor cells, mouse strain and replicate were significant factors (in varying degrees) which influenced the experimental data.

MATERIALS AND METHODS

Mice. C57BL/6 (H-2^b) and (C57BL/6 x CBA) F1 hybrid (H-2^b x H-2^k), henceforth B6CBF1, mice were obtained from Cumberland View Farms, Clinton, Tennessee. All mice used in this work were males, 8-12 weeks of age. The animals were maintained on a 6 a.m. to 6 p.m. light-dark cycle in filter covered cages. During the light portion of the cycle, mice were exposed to 75-100 foot-candles of illumination. Wayne Lab-Blox and acidified (pH 2.5) water were available ad libitum. All mice were acclimated to laboratory conditions for 2 weeks. During this time they were quarantined until they were certified free of lesions of murine pneumonia complex and of oropharyngeal Pseudomonas spp.

Tumor. The Lewis lung (3LL) carcinoma, a metastatic tumor which arose spontaneously in the pulmonary tissues of a C57BL/6 mouse,[30] was received from the NCI-NIH in its 86th sc passage in C57BL/6 male mice. In the work reported here, tumor cells were derived from the 4th-12th sc passage maintained in C57BL/6 male mice at this Institute. For routine passage, viable tumor tissue was homogenized and 0.2 ml of a 10 percent (v/v) concentration of cells in Roswell Park Memorial Institute (RPMI)-1640 medium was implanted sc. In experiments where known numbers of viable tumor cells were needed, tumor tissue was first passaged through a tissue press into RPMI-1640. Cell clumps were disrupted with a syringe fitted with an 18- and then a 20-gauge needle. Debris and dead cells were removed by layering on 100 percent fetal calf serum (Gibco, Long Island, New York) and centrifuged for 10 minutes at 750 x g. The cells were resuspended, clumps allowed to settle out, and the suspension was layered on 10-ml quantities of a Ficoll-Hypaque solution (2.4 parts of 9 percent Ficoll 400 (Pharmacia, Uppsala, Sweden) and 1 part of 34 percent Hypaque (sodium Diatrizoate, Winthrop Laboratories, New York, New York)) in glass conical tubes and centrifuged for 20 minutes at 1000 x g. The internatant layer of cells was drawn off, washed in RPMI-1640, resuspended and counted. Viability was 90-99 percent as determined by exclusion of 0.1 percent trypan blue. All tumor

cell preparations were free of bacterial contamination as determined by lack of growth in thioglycollate broth.

Groups of C57BL/6 or B6CBF1 mice were engrafted sc according to experimental needs either with 10^3, 10^4, 5×10^4 or 2×10^5 viable 3LL cells. Control groups of both mouse strains were injected sc with RPMI-1640 or 2×10^5 3LL cells given 2000 rads ^{60}Co at 600 rads/minute. When examined at necropsy, no tumor growth was observed in any of the mice engrafted with irradiated tumor cells at any of the time intervals.

Colony forming unit-spleen (CFU-s) assay. The CFU-s assay[31] was used to determine the number of stem cells found in the spleen of normal and tumor cell engrafted mice. This test was performed by the iv injection of groups of 6-8 irradiated mice each with 2×10^5 spleen cells derived from tumor cell injected mice or control treated animals. Endogenous spleen colony formation was obviated by giving B6CBF1 mice 1000 rads and C57BL/6 mice 900 rads of ^{60}Co radiation at 40 rads/minute. Irradiated mice were engrafted with syngeneic spleen cells within 4 hours of irradiation. The spleens were removed 8 days later, fixed in Bouin's solution for 2-4 hours and the surface colonies counted independently by three individuals. The average number of colonies per spleen was determined from the three counts. The number of CFU-s per 10^6 nucleated spleen cells was determined by multiplying the average number of nodules per spleen by the appropriate factor and then preparing a grand mean from the adjusted values for each treatment group.

Colony forming unit-culture (CFU-c) assay. The CFU-c assay was used to assay for granulocyte-macrophage progenitor cell population changes in the spleens of mice injected with tumor cells or mice treated as controls. Briefly, the technique consisted of a two-layer agar system using a firm 0.5 percent nutrient agar underlayer containing colony stimulating activity (CSA) and an overlayer of 0.3 percent nutrient agar containing 10^6 spleen cells per culture plate. Extracts from the placentae and uteri of pregnant mice (PMUE) were used as the source of CSA.[5] The maximum CSA was observed with a 3.3 percent

concentration (v/v) of PMUE in culture medium plus agar. Approximately 100-200 colonies/10^6 spleen cells derived from normal mice of either strain were measured at this PMUE concentration. Three replicate plates were incubated at 37°C in 5 percent CO_2. Plates containing cells derived from the spleen were counted for colonies (>50 cells) and clusters (<50 cells) after 10 days of incubation.

Mitogenic stimulation. The nonspecific thymus (T) and bone marrow (B) derived lymphocyte responses of spleen cells were determined to see if splenic enlargement in tumor cell injected mice was associated with changes in stimulation responses to mitogens. T-lymphocyte function was estimated by culture in the presence of 2.5 μg purified phytohemagglutinin (PHA-P, Wellcome Reagents Division, Research Triangle Park, North Carolina). B-lymphocyte function was ascertained by culture in the presence of 10 μg Salmonella typhosa 0901 lipopolysaccharide (LPS, Difco, Detroit, Michigan). The quantities of mitogens employed in the splenic cultures were not toxic as determined in dilution assays varying in quantity from 0.5 to 10 μg of PHA and from 1 to 20 μg of LPS. One-milliliter cultures containing 2 x 10^6 cells in RPMI-1640 medium were supplemented with 200 units of penicillin plus 200 μg streptomycin and 5 percent normal human serum (NHS) inactivated for 30 minutes at 56°C. All cell cultures were incubated in 5 percent CO_2, at 37°C and harvested at 72 hours. All spleen cultures were done in duplicate. One microcurie of tritiated thymidine (^{3}H-TdR) with a specific activity of 2.0 Ci/mmole (New England Nuclear, Boston, Massachusetts) was added to the cell cultures 18-20 hours before harvesting. Unincorporated isotope was removed by two washes in 0.9 percent saline and the cells were precipitated with 5 percent trichloroacetic acid, solubilized in NCS (Amersham/Searle Corporation, Chicago, Illinois) and counted for 1 minute in 10 ml of Spectrofluor (Amersham/Searle Corporation) cocktail. The data presented in the Tables are the counts per minute (CPM) of DNA-incorporated ^{3}H-TdR minus the CPM of the background. The background CPM is defined as the incorporation of ^{3}H-TdR into cell cultures not stimulated with mitogens.

Microscopy. Light microscopy was done on portions of the spleens of normal or tumor cell engrafted mice to correlate histologic alterations with those observations made in the cell assays. Histologic techniques employed were standard methods; staining was done on 5-μm thick sections with hematoxylin and eosin.

In the CFU-s assay, spleen colonies were identified as either erythrocytic, granulocytic or megakaryocytic. Splenic nodules were classified as mixed when an equal proportion of erythrocytic and granulocytic elements was present or when three or more megakaryocytes were found in a single section of either a granulocytic or erythrocytic nodule.

Electron micrographs were prepared on a few tumors and spleens of tumor-bearing mice to look for the presence of virus-like particles. These tissues were fixed first in 2.5 percent glutaraldehyde. The specimens were trimmed to the appropriate size and additionally fixed in 2 percent osmium tetroxide at 4°C for 2 hours. Dehydration was accomplished by serial passage through increasing concentrations of ethanol. The material was embedded in Epon, cut with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Siemens 1A electron microscope. Tissue preparations were not digested to remove glycogen particles.

Statistical methods. The experiments were designed and carried out as a three factor with two replicates array on each of the measured variates. The analyses for all variates were done on tissue from the same groups of animals so that comparisons and correlations between the quantities could be made under identical conditions.

The main effects of time, dose and strain on CFU-s, CFU-c, CPM-PHA, CPM-LPS and spleen weight were examined using an analysis of variance[29] computed with the multivariate analysis of variance (MANOVA) program.[6] The combined effects of pairs of factors (interaction) were tested to determine, for example, whether the tumor cell doses affected the two strains of mice similarly.

The analysis of variance was used since it (1) has the advantage of pooling all of the replicates to maximize the degrees of freedom in the "error" term and (2) permits the interactions to be tested.

The arithmetic of the analyses and the significance tests was computed using the square root transformation since the data followed the Poisson distribution in which the variances were proportional to the magnitude of the responses. This transformation stabilized the variances and made the data amenable to the analysis of variance technique.

All main effects of time, tumor cell dose, mouse strain and replicate and the two-factor interactions were tested for significance by pooling the higher order interactions to form the error term. When either dose or time was found significant for any variate, individual comparisons were made using the Student-Newman-Keuls test.[29] Thus, it was possible to test for differences in spleen cell responses derived from tumor cell injected mice with those responses obtained from mice not given tumor cells or from mice given irradiated tumor cells.

The significance level used throughout was $\alpha = .01$. This probability level was used for making comparisons between main effects, interactions and individual responses.

RESULTS

Tumor-cell titration. In order to determine the tumor cell doses to be used in this investigation, a 3LL cell dose-tumor growth and mortality study was done over a 60-day observation period with both C57BL/6 and B6CBF1 mice (Table 1). Resistance to 3LL tumor growth was seen in B6CBF1 mice. Thus, as compared to B6CBF1 mice (1) the numbers of C57BL/6 mice developing tumors were greater, (2) the mean times to the first palpation of tumors were shorter and (3) the mean survival times of animals dying with tumors were shorter. The times of first tumor palpation ranged from 5-21 days and times of death from the

Table 1. Tumor Incidence and Mortality in C57BL/6 and B6CBF1 Mice Engrafted With Various Quantities of 3LL Carcinoma Cells

Mouse Strain Engrafted[1]	C57BL/6			B6CBF1		
No. 3LL cells engrafted into mice	No. with tumors/ no. engrafted[2]	Time (days) of first tumor palpation[3]	M.S.T.+S.E. (days)[4]	No. with tumors/ no. engrafted[2]	Time (days) of first tumor palpation[3]	M.S.T.+S.E. (days)[4]
1×10^3	2/20	18.0 ± 0	24.0 ± 1.6	0/20	-	-
1×10^4	8/20	16.8 ± 1.9	21.8 ± 1.7	4/20	19.5 ± 1.5	22.0 ± 1.0
5×10^4	15/20	8.7 ± 0.9	27.0 ± 3.1	10/20	12.8 ± 0.5	33.4 ± 6.5
2×10^5	15/15	7.1 ± 0.7	20.5 ± 2.4	19/20	6.9 ± 0.9	28.0 ± 4.3
2×10^5 irradiated[5]	0/20	-	-	0/20	-	-
0	0/10	-	-	0/10	-	-

[1] All mice were engrafted sc with Lewis Lung carcinoma cells derived from tumors 10 days after implantation passage numbers 4 and 12 in C57BL/6 ♀ mice. Mice were palpated for tumors three times weekly starting 5 days after engraftment and continuing for 3 weeks thereafter.

[2] All tumors appeared between days 5 and 21. Experimental mice were observed for tumors for 60 days while control treated mice were examined for tumors for 30 days.

[3] Mean time in days ± standard error.

[4] M.S.T. ± S.E. = Mean survival time ± standard error of mice dying with tumors.

[5] Cells were given 2000 rads ^{60}Co at 600 rads/minute.

tumor ranged from 14-49 days. These measurements differed according to mouse strain and tumor cell load and are reflected in Table 1 by the mean values. Mice treated as controls and given either RPMI-1640 or 2×10^5 irradiated 3LL cells did not develop tumors within the 60-day observation time. At the time of injection into mice, 80-90 percent of the irradiated tumor cells excluded 0.2 percent trypan blue dye.

Splenic enlargement and histology. Splenic enlargement of the mice that died from the tumor cell titration study was noted at necropsy. This phenomenon was investigated as a function of strain of mice, time and tumor cell dose in additional groups of mice as is recorded in Table 2. Two strains of mice were employed since the tumor cell titration study supported the idea that B6CBF1 mice were somewhat more resistant than C57BL/6 mice to the 3LL carcinoma. Groups of these mice were injected with tumor cells, then sacrificed and examined 3, 7 and 14 days after engraftment to avoid morbidity from

Table 2. Splenic Weights From Two Replicates of C57BL/6 and B6CBF1 Mice as a Function of Tumor Cell Dose and Time After Engraftment [1]

Days after engraftment with 3LL tumor cells	Number of 3LL cells injected s.c. into mice					Average splenic weight (mg) per day after engraftment	Average splenic weight (mg) per day per replicate	
	0	irradiated $2x10^5$	$1x10^4$	$5x10^4$	$2x10^5$		1	2
3	77.5 ± 2.6 (4)	96.4 ± 5.4 (4)	91.9 ± 2.1 (4)	89.5 ± 6.6 (4)	91.7 ± 6.5 (4)	89.4 ± 2.5 (20)	96.0 ± 3.1 (10)	82.8 ± 2.5 (10)
7	80.1 ± 6.1 (4)	106.0 ± 9.9 (4)	97.6 ± 4.9 (4)	101.0 ± 5.2 (4)	114.1 ± 10.9 (4)	99.7 ± 4.0 (20)	107.8 ± 5.5 (10)	91.7 ± 4.9 (10)
14	74.8 ± 5.5 (4)	173.2 ± 19.7 (4)	96.9 ± 4.8 (4)	150.8 ± 14.5 (4)	235.1 ± 6.0 (4)	146.1 ± 13.9 (20)	145.0 ± 19.8 (10)	147.3 ± 20.5 (10)
Average splenic weight (mg) per tumor-cell dose	77.4 ± 2.7 (12)	125.2 ± 12.4 (12)	95.5 ± 2.3 (12)	113.8 ± 9.4 (12)	146.9 ± 19.4 (12)		116.3 ± 7.7 (30)	107.2 ± 8.6 (30)

[1] Each number is a grand mean comprised of the average of the number of mean values listed in the ( ) ± the standard error. Each individual mean value was based on measurements made on the spleens of 3-5 mice. Portions of these spleens were used to obtain the data presented in Tables 3-7.

excessive tumor growth and microbial complications. The tumor cell doses were intentionally chosen such that mice would receive either (1) a tumor cell load (2×10^5, 3LL cells) that produced tumors in nearly all injected mice or (2) intermediate tumor cell loads (1×10^4 and 5×10^4, 3LL cells) that resulted in growth characteristics that differed in the two mouse strains. Mice injected either with 2×10^5 irradiated 3LL cells or RPMI-1640 were used as controls.

The data presented in Table 2 are the average spleen weights from equal numbers of C57BL/6 and B6CBF1 mice treated as previously described. Splenic enlargement was seen in all mice injected with 3LL carcinoma cells. The degree of enlargement was dependent upon the number of tumor cells injected into mice, the condition of the tumor cells (irradiated or nonirradiated) and the time elapsed between engraftment and spleen weight measurement. Splenic weights were significantly ($p < .01$) greater at the 14-day time period than those

weights measured on day 3 and 7. The dependence of splenic enlargement upon initial tumor cell load was strongly manifested by day 14. At that time the average spleen weights of all groups of mice injected with nonirradiated cells were significantly different from each other ($p < .01$).

The average spleen weights of mice injected with tumor cells were significantly different ($p < .01$) in the two replicates reported in Table 2. There is no explanation for this observation as factors such as animal husbandry, age, sex, tumor cell preparation, etc. were similar. It should also be stated that the splenic weights depended upon the quantity of tumor cells engrafted into the mice and not upon the time of first palpation or size of the tumors.

Splenic enlargements in the two mouse strains were not significantly different (data array not presented). However, in most instances the spleens of B6CBF1 mice were heavier than those of C57BL/6 mice.

Bright light and electron microscopic tissue examinations were done to provide a possible explanation for splenic enlargement. Splenic enlargement was not associated with metastasis to the spleen because no lesions were seen in over 100 tissue samples. Although increases in splenic cell numbers were observed, these were not quantified because half of each spleen was used to prepare the cell suspensions used in the tests presented elsewhere in this report. The hypercellularity was predominantly a reticuloendothelialcytosis whose most numerous elements were macrophages. This was the most consistent finding in the spleens of all mice and was most notable in the B6CBF1 animals. Hyperplasia of the reticuloendothelial system increased over that of controls both as a function of tumor cell load and time after engraftment. Lymphoid hyperplasia was found only in C57BL/6 mice engrafted with 2×10^5 nonirradiated 3LL cells. Lymphocytosis was never seen in B6CBF1 mice. Myeloid hypercellularity was observed in both mouse strains. The most prominent myeloid activity was a megakaryocytosis that reached its apex 7 days after engraftment with tumor cells.

Cellular proliferation in the spleens of mice engrafted with 3LL cells did not seem to be associated with any unusual viral replication. C-type virus particles were not found either in representative spleen samples from control treated mice or from splenic or tumor tissue derived from mice engrafted with 3LL cells. Glycogen-like particles were observed in normal quantities in all cell types of all tissues, but glycogen digestion was not done to unequivocally substantiate the nature of this material.

Splenic myeloproliferative cells. Splenic myeloid proliferation was observed in mice engrafted with 3LL cells as was previously mentioned. The CFU-s assay was used to quantify the concentration of stem cells while the CFU-c assay was used to determine the concentration of granulocyte-macrophage progenitor cells. The data presented in Table 3 are the average number of CFU-s per 10^6 spleen cells obtained from two replicates of B6CBF1 mice and

Table 3. Number of Splenic Colonies (CFU-s) Formed Per 10^6 Spleen Cells From C57BL/6 and B6CBF1 Mice From Combined Replicates as a Function of Tumor Cell Dose and Time After Engraftment[1]

Days after engraftment with 3LL tumor cells	Number of 3LL cells injected s.c. into mice					Average number CFU-s per 10^6 spleen cells per day of engraftment	Average number of CFU-s per 10^6 spleen cells per strain	
	0	irradiated $2x10^5$	$1x10^4$	$5x10^4$	$2x10^5$		C57Bl/6	B6CBF1
3	25±4[2] (3)	32±2 (3)	22±2 (3)	30±3 (3)	27±3 (3)	27±1 (15)	27±4 (5)	27±1 (10)
7	28±4 (3)	50±4 (3)	41±7 (3)	41±3 (3)	42±6 (3)	40±3 (15)	33±4 (5)	44±3 (10)
14	32±2 (3)	29±4 (3)	37±2 (3)	43±8 (3)	38±5 (3)	36±2 (15)	28±2 (5)	39±2 (10)
Average number CFU-s per 10^6 spleen cells per dose of engrafted tumor cells	28±2 (9)	38±4 (9)	33±3 (9)	38±3 (9)	36±3 (9)		29±1 (15)	37±2 (30)

[1] The technique of determining the number of CFU-s per 10^6 spleen cells is described in the Materials and Methods.

[2] Each number is a grand mean comprised of the average of the number of mean values listed in the ( ) ± the standard error. Each individual mean value was constructed from three estimates of 6-8 mouse spleens. The cells for these assays were obtained from portions of the spleens used to obtain the data presented in Tables 2, 4-7.

one test done with C57BL/6 mice. With a few exceptions, it is observed that the CFU-s concentration in spleen cells obtained from mice injected with 3LL cells is greater than controls. This increase in concentration was significant ($p < .01$) and appears to be dependent upon the initial tumor cell load, time of assay after tumor cell implantation and the strain of mouse injected with tumor cells. The concentration of CFU-s per 10^6 spleen cells, with one exception, tended to be the greatest on day 7 after engraftment with 3LL cells. The splenic concentration of CFU-s was greater ($p < .01$) in B6CBF1 than in C57BL/6 mice after injection with 3LL cells. However, the percentage increases over control treated C57BL/6 (22 ± 4 CFU-s/10^6) and B6CBF1 (31 ± 4 CFU-s/10^6) mice were similar, as the CFU-s concentration increased 10-15 percent in each strain after transplantation with 3LL cells.

Spleen colony histologic identification was done only on B6CBF1 mouse spleens. Approximately three spleens per time interval per replicate per tumor cell dose (90 spleens with about 400 colonies) were examined. About 1 percent of the colonies were diagnosed as being of mixed granulocytic-erythrocytic composition. A ratio of three erythrocytic colonies for every granulocytic colony was found for both control treated and tumor cell injected mice.

The data presented in Table 4 are the average number of CFU-c per 10^6 spleen cells obtained from unequal groups of C57BL/6 and B6CBF1 mice. The CFU-c concentration of spleen cells obtained from mice injected with 3LL cells was increased over that observed for spleen cells from mice not given 3LL cells. Successively higher 3LL tumor cell inocula resulted in progressively increased numbers of CFU-c at each time interval tested. There was a tendency for the CFU-c concentrations of spleen cells obtained from mice injected with the two highest doses of tumor cells to increase over time. The concentration of CFU-c in the spleens of B6CBF1 mice tended to be greater than that found in C57BL/6 mice. Cluster formation was found in culture plates seeded with cells obtained from mice 2 weeks after injection with either of the highest number of tumor cells. Cluster formation results from the limited proliferation of differentiated

Table 4. Number of Soft Agar Colonies (CFU-c) Formed Per 10^6 Spleen Cells From C57BL/6 and B6CBF1 Mice From Combined Replicates as a Function of Tumor Cell Dose and Time After Engraftment[1]

Days after engraftment with 3LL tumor cells	Number of 3LL cells injected s.c. into mice					Average number CFU-c per 10^6 spleen cells per day of engraftment	Average number of CFU-c per 10^6 spleen cells per strain	
	0	*irradiated* $2x10^5$	$1x10^4$	$5x10^4$	$2x10^5$		C57Bl/6	B6CBF1
3	24±3[2] (2)	61±33 (2)	37±1 (2)	40±0 (1)	83±26 (2)	50±10 (9)	36±8 (4)	61±17 (5)
7	20±9 (3)	66±31 (3)	31±13 (3)	60±32 (3)	81±40 (3)	51±12 (15)	61±15 (5)	80±26 (5)
14	19±5 (4)	100±15 (4)	24±6 (4)	78±13[3] (4)	102±17[3] (4)	65±10 (20)	52±16 (5)	48±14 (5)
Average number CFU-c per 10^6 spleen cells per dose of engrafted tumor cells	20±3 (9)	80±14 (9)	29±5 (9)	67±13 (8)	91±15 (9)		59±9 (19)	55±9 (25)

[1,2] Footnotes from Table 3 apply to the data presented in Table 4.

[3] Uncountable numbers of clusters were found at this cell dose and time interval.

precursor cells. The cellular morphology of all colonies was of mononuclear, monocyte-macrophage type cells. No granulocytes were observed in any colonies at any stage of growth.

Splenic cell responses to mitogens. Hyperplasia of the splenic myeloid cell compartment with some expansion of the lymphoid cell system was seen in mice injected with 3LL cells. Thus, it was hypothesized that increased splenic cell proliferation would result in the increased incorporation of ^{3}H-TdR in mitogen unstimulated cells as well as alterations in isotope uptake of lymphocytes stimulated either with PHA or LPS. Analyses for (1) naturally proliferating cells, (2) T-lymphocytes and (3) B-lymphocytes were done on spleen cell samples at the same time to test this hypothesis.

Presented in Table 5 are the average CPM of unstimulated spleen cell cultures derived from equal numbers of C57BL/6 and B6CBF1 mice treated as previously described. The average ^{3}H-TdR incorporation into the spleen cells of all mice injected with 3LL cells was significantly increased over that isotope

Table 5. Counts Per Minute (CPM) of Unstimulated Spleen Cell Cultures From Two Replicates of C57BL/6 and B6CBF1 Mice as a Function of Tumor Cell Dose and Time After Engraftment[1]

Days after engraftment with 3LL tumor cells	Number of 3LL cells injected s.c. into mice					Average CPM per day of engraftment	Average CPM per Replicate number	
	0	Irradiated $2x10^5$	$1x10^4$	$5x10^4$	$2x10^5$		1	2
3	16,546± 4,384[2] (4)	30,619± 7,774 (4)	24,710± 2,162 (4)	21,718± 2,153 (4)	23,937± 7,002 (4)	23,506± 2,335 (20)	22,133± 3,409 (10)	24,879± 3,313 (10)
7	4,726± 839 (4)	14,508± 2,941 (4)	9,457± 3,151 (4)	15,251± 6,694 (4)	13,607± 2,900 (4)	11,510± 1,825 (20)	6,785± 998 (10)	16,235± 2,847 (10)
14	5,031± 1,435 (4)	18,710± 4,842 (4)	10,402± 4,006 (4)	30,350± 7,254 (4)	33,164± 4,590 (4)	19,531± 3,150 (20)	15,228± 3,164 (10)	23,834± 5,270 (10)
Average CPM per engrafted tumor-cell dose	8,768± 2,181 (12)	21,279± 3,645 (12)	14,856± 2,686 (12)	22,440± 3,571 (12)	23,506± 3,597 (12)		14,715± 1,923 (30)	21,649± 2,315 (30)

[1] The technique of cell culturing is described in the Materials and Methods.

[2] Each number is a grand mean comprised of the average of the number of mean values listed in the ( ) ± the standard error. Each individual mean value was constructed from duplicate cell cultures made from portions of the spleens used to obtain the data presented in Tables 2, 3, 4, 6 and 7.

uptake measured for spleen cells taken from mice not injected with tumor cells. Overall, the uptake of isotope was dependent upon the factors of time, dose of tumor cells and replication. Generally, the isotopic uptake of mitogen unstimulated cells reached (1) a nadir 7 days after the mice were injected with 3LL cells and (2) its highest level at day 14 in mice injected with the two greatest numbers of nonirradiated 3LL cells. The reason for the large differences in isotope uptake between the two replicates on days 7 and 14 is not known.

The data presented in Table 5 support the contention that spleen cells from mice injected with 3LL cells contain a significant component of replicating cells capable of incorporating ^{3}H-TdR without the addition of a mitogen. Consequently, the uptake of isotope due to mitogenic stimulation was corrected by subtracting the appropriate "background" obtained from unstimulated cell cultures. The data

presented in Table 6 then are the corrected CPM of spleen cells incorporating ^{3}H-TdR after PHA stimulation. Overall, the PHA response of splenic lymphocytes obtained from C57BL/6 and B6CBF1 mice injected with 3LL cells was less than that of splenic lymphocytes obtained from control treated mice. The uptake of ^{3}H-TdR by splenic lymphocytes was progressively decreased as the number of 3LL tumor cells injected into the mice was increased. The length of time spleens were in a 3LL cell injected mouse had no significant effect upon the incorporation of ^{3}H-TdR in PHA-stimulated T-lymphocytes. C57BL/6 mouse splenic lymphocytes had a significantly ($p < .01$) reduced uptake of ^{3}H-TdR after PHA stimulation as compared to that ascertained for cells obtained from B6CBF1 mice.

The B-lymphocyte component of spleens obtained from mice injected with 3LL cells was investigated simultaneously with that of the previously mentioned T-lymphocyte responses. The data presented in Table 7 as corrected CPM

Table 6. Counts Per Minute (CPM) of PHA-Stimulated Spleen Cell Cultures From Combined Replicates of C57BL/6 and B6CBF1 Mice as a Function of Tumor Cell Dose and Time After Engraftment[1]

Days after engraftment with 3LL tumor cells	Number of 3LL cells injected s.c. into mice					Average CPM per day of engraftment	Average CPM per strain	
	0	Irradiated $2x10^5$	$1x10^4$	$5x10^4$	$2x10^5$		C57Bl/6	B6CBF1
3	534,476± 46,807[2] (4)	374,359± 36,876 (4)	303,137± 73,725 (4)	322,721± 55,923 (4)	245,751± 65,637 (4)	356,089± 32,038 (20)	313,855± 34,365 (10)	398,323± 52,503 (10)
7	494,323± 60,719 (4)	440,059± 56,737 (4)	404,937± 38,348 (4)	413,655± 27,297 (4)	360,184± 65,905 (4)	422,632± 22,972 (20)	377,813± 32,604 (10)	467,451± 26,808 (10)
14	509,974± 38,635 (4)	388,848± 59,294 (4)	480,648± 50,107 (4)	401,990± 44,977 (4)	241,761± 39,614 (4)	404,644± 29,078 (20)	355,537± 43,591 (10)	453,751± 33,673 (10)
Average CPM per engrafted tumor-cell dose	512,924± 26,360 (12)	401,089± 28,427 (12)	396,241± 36,564 (12)	379,455± 28,023 (12)	282,565± 34,688 (12)		349,068± 21,282 (30)	439,841± 22,530 (30)

[1,2] Footnotes 1 and 2 from Table 5 apply to the data presented in Table 6.

Table 7. Counts Per Minute (CPM) of LPS-Stimulated Spleen Cell Cultures From Combined Replicates of C57BL/6 and B6CBF1 Mice as a Function of Tumor Cell Dose and Time After Engraftment[1]

Days after engraftment with 3LL tumor cells	Number of 3LL cells injected s.c. into mice					Average CPM per day of engraftment	Average CPM per strain	
	0	Irradiated $2x10^5$	$1x10^4$	$5x10^4$	$2x10^5$		C57Bl/6	B6CBF1
3	53,220± 8,712[2] (4)	51,641± 8,088 (4)	56,615± 7,534 (4)	56,179± 6,014 (4)	42,661± 12,089 (4)	52,063± 3,655 (20)	48,492± 4,516 (10)	55,634± 5,759 (10)
7	44,843± 8,855 (4)	55,306± 11,983 (4)	50,691± 17,332 (4)	41,973± 12,256 (4)	46,497± 13,524 (4)	47,862± 5,307 (20)	34,760± 5,232 (10)	60,964± 7,307 (10)
14	40,739± 12,249 (4)	66,187± 20,545 (4)	59,832± 11,750 (4)	69,703± 23,541 (4)	43,376± 10,432 (4)	55,968± 7,122 (20)	47,749± 10,277 (10)	64,186± 9,670 (10)
Average CPM per engrafted tumor-cell dose	42,267± 5,488 (12)	57,712± 7,800 (12)	55,713± 6,806 (12)	44,952± 8,887 (12)	44,178± 6,329 (12)		43,667± 4,152 (30)	60,262± 4,365 (30)

[1,2] Footnotes 1 and 2 from Table 5 apply to the data presented in Table 7.

support the contention that there is an increased B-lymphocyte response in mice injected with 3LL cells. The incorporations of ^{3}H-TdR by LPS stimulated B-lymphocytes derived from mice injected with different amounts of 3LL cells were not significantly different from mice not injected with tumor cells. While not statistically different it is of interest that the highest tumor cell dose resulted in the smallest increase in ^{3}H-TdR incorporation into LPS stimulated splenic lymphocytes. There was, however, a significant difference ($p<.01$) in LPS stimulated lymphocyte incorporation of ^{3}H-TdR between the two mouse strains. That is, as in the case of the T-lymphocyte response, spleen cell cultures made from B6CBF1 mice incorporated more isotope than cultures made from C57BL/6 mice.

DISCUSSION

The significant findings of this work include (1) the differential tumor growth responses in mice engrafted with 3LL carcinoma cells, (2) hyperplasia of splenic myeloid and reticuloendothelial elements resulting in splenomegaly, (3) splenomegaly associated with greater concentrations of CFU-s and CFU-c and (4) splenomegaly associated with a decreased response to PHA and a marginally increased response to LPS. In addition, all of the cell assay systems employed to delineate the 3LL-induced splenomegaly were dependent, to some extent, upon (1) the initial dose of tumor cells, (2) time after engraftment when the spleen cells were used for the assays, (3) strain of mouse injected with 3LL cells and (4) experimental replication.

The differential tumor growth responses and splenomegaly responses in C57BL/6 and B6CBF1 mice were unexpected findings. A tempting explanation for these observations, supported by our histologic findings of increased numbers of splenic macrophages in B6CBF1 mice, is that the 3LL cells provoked a nonspecific host resistance to tumor growth. This explanation for resistance to tumor growth is supported by the observations that (1) while splenic macrophages were increased in 3LL cell-injected C57BL/6 mice, their numbers were greater in B6CBF1 mice, (2) large numbers of macrophages in the tumor area are positively correlated with a good prognosis after surgical extirpation[4] and (3) agents that stimulate macrophage reactivity may be useful as immunologic adjuvants.[20]

Splenomegaly in animals bearing transplantable nonhematogenous derived tumors has been explained by several investigators as the result of microbial complications.[2,8] This conclusion is not supported by our observations since (1) unusual viral activity was not observed, (2) cultures of spleen cells never resulted in detectable bacterial growth, (3) ulceration of tumors was not seen during the time period investigated in this report, (4) injection of mice with sterile irradiated 3LL cells resulted in splenomegaly and (5) splenomegaly was observed in mice reared under axenic conditions and grafted with sterile preparations of a transplantable myoepithelioma.[12]

It was not possible to estimate the total content of CFU-s and CFU-c in the spleens of mice injected with 3LL cells because a portion of each spleen was used for histologic evaluation. Nevertheless, increases in CFU-s and CFU-c concentrations within the spleen of 3LL cell-injected mice were observed. A number of factors operating either independently or together may account for the increased CFU-s and CFU-c concentrations in the spleen. These include increases due either to (1) stimulatory factors released by the 3LL cells, (2) responses to antigenic stimulation provided either by tumor cell debris or possible tumor specific antigens (TSA) or (3) the migration or mobilization of such cells from other hematopoietic tissues such as the bone marrow to the spleen. A fourth tenable hypothesis is that the fraction (f) of CFU-s seeding in the spleen of the irradiated assay mouse was altered.[27,28] This hypothesis merits investigation, since a number of substances known to perturb the total CFU-s compartment result in variations in the fraction of CFU-s capable of seeding the spleens of irradiated assay mice.[21] It is of interest to note that if this fraction is lower for spleen cells derived from mice injected with 3LL cells than it is for mice not injected with 3LL cells, the values presented in Table 3 are underestimates of the splenic CFU-s concentration. The fraction of CFU-c capable of seeding on agar plates is assumed to be "100 percent" as such cells are not able to be recultured.

An erythrocytic-granulocytic (EG) ratio of three was found for colonies obtained from the cells of spleen derived from either control treated or 3LL cell-injected mice. This ratio is commonly found in many mouse strains where bone marrow cells are the source of the CFU-s.[35] When normal spleen cells are the source of the CFU-s, an EG ratio two to ten times greater than that for marrow cells is observed.[34] Because of similar EG ratios found for spleen cells derived from either control treated or 3LL cell-injected mice, it is not possible to state unequivocally that migration of CFU-s occurred from the marrow to the spleen.

Hematocytopoietic alterations produced by certain tumor-related substances have been identified previously. A granulocytosis promoting factor was isolated from a transplantable mouse mammary tumor.[9] Colony stimulating factor (CSF), a substance capable of stimulating CFU-c growth, has been found in the serum and urine of leukemic individuals.[21] Identification of a somewhat similar substance produced by 3LL tumor cells is a distinct possibility in light of the data reported here.

Antigenic substances are known to increase the splenic numbers of CFU-s and CFU-c.[19,26] Debris from tumor cell destruction or tumor specific antigens then could provoke increases in these cell types. Recognition of the tumor cell debris could also explain the large number of macrophages seen in the spleens of B6CBF1 mice injected with 3LL cells. In vitro sensitization of C57BL/6 lymphocytes to 3LL cells supports the contention that TSA are present on the tumor cell membranes.[33] In addition, the H-2^b specificity of the original C57BL/6 host mouse may be carried on 3LL tumor cell membranes.[32] Thus, the findings of others support the hypothesis that some form of antigenicity may be responsible for a portion or all of the increased splenic concentrations of CFU-s and CFU-c.

In mice injected with 3LL cells, spontaneous incorporation of ^{3}H-TdR up to six times that found in control treated animals was observed. It is tempting to ascribe these increases to proliferation of cells within the myeloid cell compartment. This is supported by our findings of (1) histologic evidence of myeloid cell proliferation, (2) the increases found in the concentrations of CFU-s and CFU-c and (3) large numbers of clusters formed from spleen cells of 3LL cell-injected mice that also had the highest spontaneous incorporation of ^{3}H-TdR (Tables 4 and 5). The contribution of spontaneous isotopic uptake into lymphocytes, proliferation of which could occur in response to tumor-cell associated antigens, seems minimal since our histologic examinations did not reveal amplification of this cell compartment.

Reductions in splenic cell-lymphoid cell responsiveness to PHA is a well-known observation. The reduced splenic cell responsiveness to PHA observed with cells obtained from mice injected with 3LL cells may be explained by any one or all of the following considerations. These are (1) dilution of the number of lymphoid cells cultured by increased myeloid cell concentrations, (2) the presence of cells or factors which inhibit T-lymphocyte responsiveness and (3) T-cell commitment to other antigens.

The data presented in this report support the contention that myeloid cell increases in the spleen cell cultures could result in decreased uptake of ^{3}H-TdR after PHA stimulation. It should be noted that while cell isotope counts were corrected for background, which would include uptake in replicating stem and progenitor cells, some of these cells would be in a resting state. Increased concentrations of such cells would then dilute the number of lymphocytes available to respond to PHA.

The histologic and CFU-c findings reported here of greater numbers and commitment towards macrophage development support the idea that these cells may be responsible for the reduced splenic lymphoid cell uptake of ^{3}H-TdR after PHA stimulation. Indeed, it was recently reported that thymidine released from cells degraded by macrophages may be responsible for suppression of ^{3}H-TdR incorporation after PHA stimulation.[14,24] In addition, tumor cells are known to release immune suppressive factors[25] but we did not test for the presence of such a material in our 3LL cell-injected mice.

Spleen cells, derived from C57BL/6 mice on days 3, 7 and 14 after injection with the various numbers of 3LL cells used in this work, were capable of inducing graft versus host disease (GVHD). Thus, all B6CBF1 mice irradiated with 1000 rads and given 5×10^{6} spleen cells from C57BL/6 mice treated either as controls or given 3LL cells died 8-14 days after transplantation. These data tend to support the argument that in an <u>in vivo</u> assay, the T-lymphocytes were immunologically capable of responding to transplantation antigens. Full T-cell commitment to tumor cell associated antigens then does not appear to be

an attractive explanation for reduced splenic cell responsiveness to PHA.[1] If commitment to such antigens had developed, mortality from GVHD would have been delayed past the 8- to 14-day survival times.

The splenic B-lymphocyte response to LPS in mice injected with 3LL cells was marginally increased over that of control treated mice. This is contrasted to the greatly enhanced splenic cell response to LPS observed for mice bearing methylcholanthrene (MCA) induced fibrosarcomas.[17] Possible explanations for the differences in our data and that of the latter authors are (1) a different tumor type was used, (2) the number of tumor cells used was 25-fold greater than that used in our work and (3) spleen cells were obtained for testing 3-8 weeks after inoculation with MCA-induced tumor cells.

In conclusion then, engraftment of mice with 3LL carcinoma cells produces splenomegaly. The splenomegaly is characterized by a reticuloendothelial-cytosis, increases in the concentration of CFU-s and CFU-c, possible decreases in lymphocyte responsiveness to PHA and marginal increases in lymphocyte responsiveness to LPS. These findings may be of value in experiments of a therapeutic nature designed to cure mice bearing the 3LL carcinoma.

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